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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(b)(2).

Docket Number	U022 1120.P1	Type a plus sign (+) inside this box →	+

INVENTOR(s)/APPLICANT(s)

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
EVANS	**Donald**	**L.**	Athens, GA
KAUR	**Harjeet**		Athens, GA
JASO-FRIEDMANN	**Liliana**		Athens, GA
LEARY	**John**		Athens, GA
PRAVEEN	**Kesavannair**		Athens, GA

TITLE OF THE INVENTION (280 characters max)

TELEOST ANTIMICROBIAL AND CYTOTOXIC POLYPEPTIDES

CORRESPONDENCE ADDRESS

WOMBLE CARLYLE SANDRIDGE & RICE
P.O. BOX 7037

STATE	ATLANTA, GEORGIA	ZIP CODE	30357-0037	COUNTRY	USA

ENCLOSED APPLICATION PARTS (*check all that apply*)

☒ **Specification** -30 Pages ☐ **Drawing(s)** *Number of Sheets* ☐ **Other (specify)**_____		
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (*check one*)		
☒ A check or money order is enclosed to cover the filing fees ☒ The Commissioner is hereby authorized to charge any deficiency in fees or credit any overpayment to Deposit Account Number: 09-0528.	FILING FEE AMOUNT ($)	**$80.00**

☒ Pursuant to 37 C.F.R. § 1.27, applicant hereby asserts small entity status.

The Invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No

☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE DJ Hayzer

Date February 18, 2004

TYPED or PRINTED NAME: David J. Hayzer, Ph.D., J.D.

REGISTRATION NO. (*if appropriate*) 43,329

☐ Additional inventors are being named on separately numbered sheets attached hereto

PATENTS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:)
EVANS et al)

For: **TELEOST ANTIMICROBIAL AND CYTOTOXIC POLYPEPTIDES**

CERTIFICATE OF EXPRESS MAIL

Mail Stop Provisional Patent Application
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Sir:
Enclosed for filing in the above case are the following documents:

Provisional Application for Patent Cover Sheet
30 Pages of Specification
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(404) 872-7000 (Telephone)
(404) 888-7490 (Facsimile)
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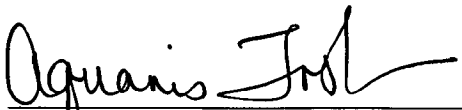
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1. A nucleic acid molecule encoding the polypeptide, a variant or fragment thereof, as shown in Fig.2.

2. A polypeptide, a variant or fragment thereof, isolated from a fish, wherein the polypeptide is capable of inhibiting the proliferation of a microorganism.

3. A method of inhibiting the proliferation of a microorganism by contacting said cell with a polypeptide according to Claim 2.

Introduction

Naturally occurring antimicrobial proteins and peptides (AMP) have been identified from a wide diversity of plant, invertebrate and vertebrate species (Hancock and Brown 1995; Hancock and Diamond 2000; Hanson et al. 2000; Vizioli and Salzet 2002; Zhai and Saier 2000). These AMP have been classified based on both chemical and conformational properties. Groups or representatives of different major categories of these substances may be differentiated based on whether the active form is a peptide (i.e. 17-35 aa in length) or a protein (>50 aa). Additional distinguishing properties of AMP are: most are cationic with little to no amino acid sequence identity across all other the members of this very large group. For example, cecropins, magainins and defensins from silk moth, *Xenopus* and mammals (respectively) are low mw AMP, all are lysine rich and inducible. However, they share no sequence homology. The functional characteristic that most unifies this large group of AMP is based on their common ability to kill bacteria and (in some cases) eukaryotic cells.

Knowledge of the amino acid content of these AMPs does signal clues regarding the common chemical and physical features that may be responsible for their bacteriocidal effects. An example is a recently described AMP (Cupiennin-1) (Kuhn-Nentwig et al. 2002). This AMP is present in the venom of *Cupiennius salei* (a hunting spider found in Central America). It is a 35 amino acid basic peptide (has 8 lysine residues) that is amphipathic and has bacteriocidal activities against Gram negative and Gram positive bacteria. This peptide may be similar to other AMPs (e.g. magainins; Jacob and Zasloff 1994) regarding the mechanism of binding to bacterial cells. It was predicted to fold into an amphipathic alpha-helix when it inserts into the bacterial cell membrane. Differential sensitivities of eukaryotic versus prokaryotic cells are

thought to be based on the low cholesterol content and relatively high negative charge density of bacterial cell walls compared to eukaryotic cells.

Although a second type of AMP cannot be considered as "natural" (i.e. they are generated *in vitro* by proteolytic digestion or acid hydrolysis of some precuror or larger mw molecule), none-the-less these AMP are relevant innate immune response effector substances. One interesting class has been studied in species ranging from teleosts to humans and is composed of histone like proteins. The traditional cellular location of histone proteins (H1) is in the nucleus associated with chromatin fibers either in the form of linker histone 1 or core histones (H2a, H2b, H3 and H4) that form nucleosomes. However, studies performed in higher vertebrates have shown that many cells of the immune system express cytoplasmic and membrane forms of these proteins. Human monocytes express membrane histones H2a and b (Bennett et al. 1985; Holers and Lotzin 1985; Emlen et al. 1992); a human transformed B cell line, Raji, expresses 14-18 and 33-34 kDa histone-like membrane proteins (Kubota et al. 1990); and T-cells express membrane H2b (17 kDa) (Ojcius et al. 1991; Watson et al. 1994) and H3 (29 kDa) (Watson et al. 1994; Watson et al. 1995). Examples of the relatively widespread expression of membrane histones are neurons (Bolton and Perry 1997) and macrophages (Brix et al. 1998) that express 30-33 kDa histone H1 membrane receptors that bind LPS and thyroglobulin, (respectively).

In teleosts, antimicrobial proteins and peptides with molecular characteristics of histones have been isolated from salmon blood, liver, intestine and mucus (Richards et al. 2001; Patrzykat et al. 2001). Catfish skin, epithelial cells and mucus contain H2a-like (Parasin-I) and H2b-like molecules (Park et al. 1998; Robinette et al. 1998). These studies demonstrated that histone release from cells required tissue injury and thus, membrane expression of histone-like proteins

was not determined. In the present study, a naturally occurring novel membrane protein referred to as NCC antimicrobial protein-1 (ncamp-1) was identified. It binds bacterial DNA and as such is postulated to participate in innate antimicrobial immunity. To study the phylogenetic and evolutionary significance of expression of this protein, ncamp-1 was sequenced and the recombinant form examined for bacteriocidal activity. Among the molecular properties of ncamp-1, it shares some sequence homology with histone 1X. A repeat amino acid sequence composed of a lysine box (i.e. lysine box motif/LBM) was identified within ncamp-1. Phylogenetic analysis and sequence comparisons with bactericidal peptides from bacteria to mammals indicated that this motif was conserved and may be responsible for elicitation of antimicrobial activities of these various peptides.

Materials and Methods

Experimental animals and isolation of NCC.

Channel catfish weighing 20-60g were net captured and sacrificed by submersion in anesthetic (3-aminobenzoic acid ethyl ester; #D-5040 Sigma). Anterior kidney (AK) tissue (mammalian bone marrow equivalent) was removed aseptically and passed through screen mesh to obtain single cell suspensions in complete RPMI-1640 containing 10% FBS. Red cells were first removed by one cycle of centrifugation through Ficoll-Hypaque, and NCC were purified by density gradient centrifugation over a 45.5 % Percoll cushion. Cells at the interface were collected, washed once with RPMI and resuspended in complete RPMI.

Preparation of cell membranes.

For membrane preparation, cells were washed three times with ice cold TBS (25mM Tris-Cl, pH 7.5, 150 mM NaCl). Cells were resuspended in Dounce homogenization buffer (10mM Tris-Cl, pH 7.6, 0.5mM $MgCl_2$, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 1mM

PMSF) @ 2 x 10^7 cells/ml and incubated on ice for 15 min. 333 µl of tonicity restoration buffer (10mM Tris-cl, pH 7.6, 0.5mM $MgCl_2$ and 0.6M NaCl) was added per ml of homogenization buffer and cells were spun at 500g for 5 min. Supernatant was collected and EDTA was added to 5mM. Supernatant was then spun at 13000 rpm for 10 min. Supernatant was discarded and pellet equivalent to1 x 10^6 cells was washed twice with cold TBS containing 10 µg/ml leupeptin, 10 µg/ml pepstatin and 1mM PMSF and finally resuspended in 100 µl of hot 1X SDS-sample buffer.

Southwestern blotting and ligand precipitation

Membrane proteins from NCC were resolved on a 12.5% SDS-PAGE and transferred on to nitrocellulose membrane. Nitrocellulose membrane was incubated in blocking buffer (Super-bloc, Pierce, Rockford, IL) containing 0.1% Tween-20 for 30 minutes, followed by 60 minutes incubation in biotinylated GpC ODN. Membranes were washed four times (5 minutes each) in TBS containing 0.1% Tween-20 and incubated for 60 minutes in ExtrAvidine-Peroxidase conjugate (diluted 1:200000 in blocking buffer). The proteins binding to the biotinylated ODN were detected with chemiluminescent substrate (SuperSignal®, West Pico Chemiluminescent, Pierce, Rockford, IL).

Ligand precipitations were done using GpC-biotin and NCC cell lysates (equivalent to 2 x 10^7 cells) at 4C for 3 hours. This mixture was then transferred to tubes containing 25 ul of ImmunoPure^R Imobilized Avidin beads (#20219 Pierce, Rockford, IL) and incubated at 4C for 1 hour. Beads were washed four times with CHAPS lysis buffer and bound proteins eluted with (reducing) SDS-PAGE sample buffer. The eluate was analyzed by SDS-PAGE followed by Southwestern blot examination as previously explained. The control consisted of probing with the ExtrAvidine-Peroxidase conjugate only.

Protein fingerprinting, primer design and PCR amplification

Proteins identified to bind to ODN in Southwestern blot were excised and protein fingerprinting was done by microcapillary reverse phase HPLC followed by ion trap mass spectrometry (MS) (Harvard Microchemistry facility). The MS spectra of peptide fragments were compared (using an algorithm called Sequest) and the results were manually verified by checking the fidelity of the run and biological significance. One of the peptide fragments identified had high degree of similarity to the MS spectra of a peptide fragment from histone H1 from trout. This peptide fragment was used to design degenerate primers to amplify portions of the gene (in combination with vector specific primers for the library) using cDNA library constructed from NCC purified from catfish anterior kidney as a template. The amplicons were cloned in to a pDrive TA cloning vector (Qiagen, Carlsbad, CA) and sequenced in a 373 A DNA sequencer (Applied Biosystems, Foster City, CA) at the Molecular Genetics Instrumentation Facility (University of Georgia, Athens) using the standard protocol described by the manufacturer. Sequences were compared with the known sequences in DDBJ/EMBL/GenBank databases using BLAST version 2.2.5 (Altschul et al. 1997). Based on the sequence, which was similar to H1 histone family X members, non-degenerate primers were designed to screen the cDNA library using a directed PCR-based iterative cloning protocol based on published procedures (Heaton et al, 1997). Several clones were sequenced in both the directions to verify the complete sequence.

Recombinant protein

Primers were designed to amplify the entire coding region of ncamp-1 to generate the recombinant protein. PCR amplified and restriction digested insert DNA was directionally

cloned in to pET-21 b expression plasmid (Novagen, San Diego, CA), which allow the expression of protein with C-terminal His-Tag. The resulting plasmid (pET-21b-ncamp) was electroporated into *E. coli* Expression strain BL21(DE3)pLysE (Novagen). Bacteria was grown till 0.6 – 1.0 OD and induced with 1mM isopropyl-beta-D-thiogalactopyranoside (IPTG, # BP1620-1, Fisher, Fair Lawn, NJ) for 3 h at 30°C. Lysates were prepared from IPTG induced cultures by sequential incubations in lysozyme (1mg/ml), Triton X-100 (0.5%), DNaseI (5 μg/ml) and RNase A (10 μg/ml). Ncamp-1 was purified from cleared lysates using Ni-NTA-agarose (#30210, Qiagen, Valencia, CA) according to manufacturer's instructions.

Antimicrobial assay

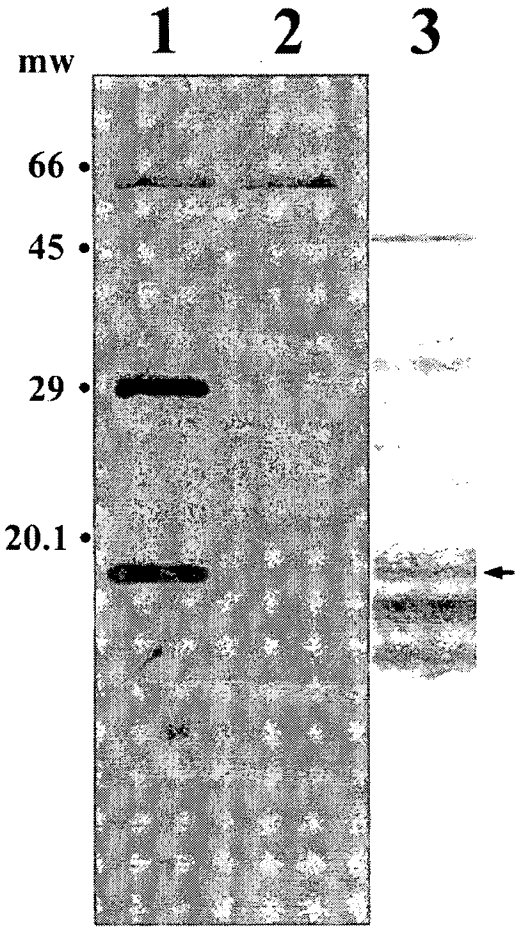
The recombinant protein was examined for anti-microbial activity by an *in vitro* bactericidal assay (Hiemstra et al. 1996) using Gram positive bacteria *Micrococcus luteus*. For this, *Micrococcus* was grown overnight at 37° C in Mueller-Hinton broth. Overnight cultures (500 ul) were diluted 1:100 in fresh broth and incubated for 2 hours at 37C followed by measuring the OD_{620} of the solution. A reading at this OD of 0.1 was equal to 1.2×10^8 colony forming units (CFUs)/ml. Bacteria were then diluted to 10^5 CFUs/ml in sodium phosphage buffer (pH 8) supplemented to 10% with MH broth (assay buffer). For the assay, different concentrations of ncamp-1 or media control (equal concentrations of bovine serum albumin) were mixed with 5000 CFUs in a final volume of 100 ul and incubated at 37° C for 2 hours with shaking. Serial dilutions of each sample were plated onto MH plates and colony counts were determined. Colony counts were expressed as a percent of media control growth. ODNs added in the absence of recombinant protein had no effect on the growth of bacteria.

Results and Discussion

Purified catfish NCC were activated by *in vitro* incubation with CpG olideoxynucleotides for 24 hours as previously described (Oumouna et al. 2002). Cell lysates of activated and resting NCC were analyzed on SDS-PAGE (12.5%) and blotted onto nitrocellulose. Membranes were probed with biotinylated GpC to identify the bacterial DNA binding proteins (Figure 1, lane 1). A band of approximate molecular weight of 18-20 kDa in the SDS-gel (Figure 1, lane 3 arrow) from activated NCC lysates was excised and sent to Harvard Microchemistry laboratory for protein fingerprinting analysis. One of the peptides identified had the following sequence: GASGSFKLNKK, and was used to design degenerate primers. Catfish NCC cDNA library was screened to identify individual clones with full-length of the gene coding for this novel protein and sequenced in both directions. The complete sequence of the protein was submitted to NCBI (accession number AY324398) and is shown in Figure 2. Unlike the histone mRNAs, this novel gene has a typical polyadenylation signal and poly-A tail, indicative of its extra-nuclear localization in cells. The open reading frame product predicts a protein of 22,064.63 Daltons and it is composed of 203 amino acids. This protein has a pI of 10.75; it is composed of 58 strongly basic amino acids (K, R); 55 hydrophobic amino acids (A, I, L, F, W, V); and it has 50 polar amino acids (N, C, Q, S, T, Y). A database search for other proteins with similar and/or identical sequences to this novel protein revealed that it is similar to H1 histone family X proteins from human, mouse, and *Xenopus* (Figure 3 and Table 1). The boxed area in Figure 3 is the source for design of the original degenerate primers. A search of the zebrafish sequences in the NCBI database revealed that a similar protein, but with "unknown" functions has been documented (accession number AAH47192). This protein is 51% similar to the novel catfish protein (Figure 4 and Table 2) and this comparison confirmed the existence of similar proteins

in other teleost species. Expression of this novel protein in other hematopoietic cell lines and tissues was verified by searching channel catfish EST database and this gene expression is reported in NK like cell line MLC-52-1 (accession numbers CB937576 and CB937396), brain (accession number BM495146) and anterior kidney (accession number BE469379).

Fig 1. Southwestern blot analysis of GpC ligand precipitated surface membrane proteins from channel catfish anterior kidney NCC. Lysates were precipitated with GpC-biotin and immobilized Streptavidin as described in the Materials section. Precipitated protein was probed with GpC-biotin (lane 1) or conjugate only (lane 2). Lane 3 is the total protein stain of the precipitate. The arrow points to the protein band that was excised and identified.



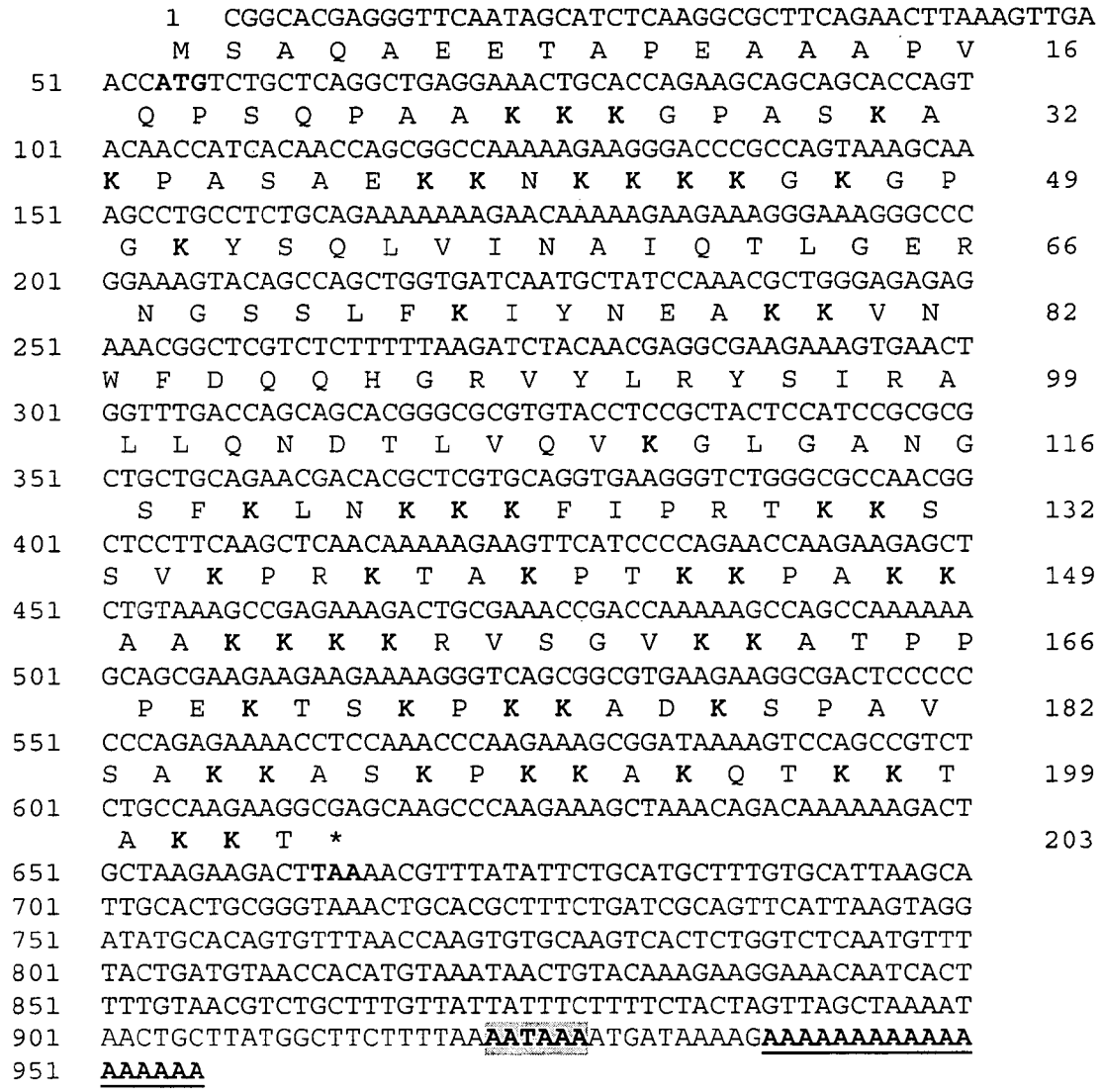


Fig. 2. Compiled full-length catfish ncamp-1 cDNA sequence. Lysine residues are represented in bold letters. Polyadenylation site is highlighted and poly A tail is underlined. Start and stop codons also are represented in bold letters.

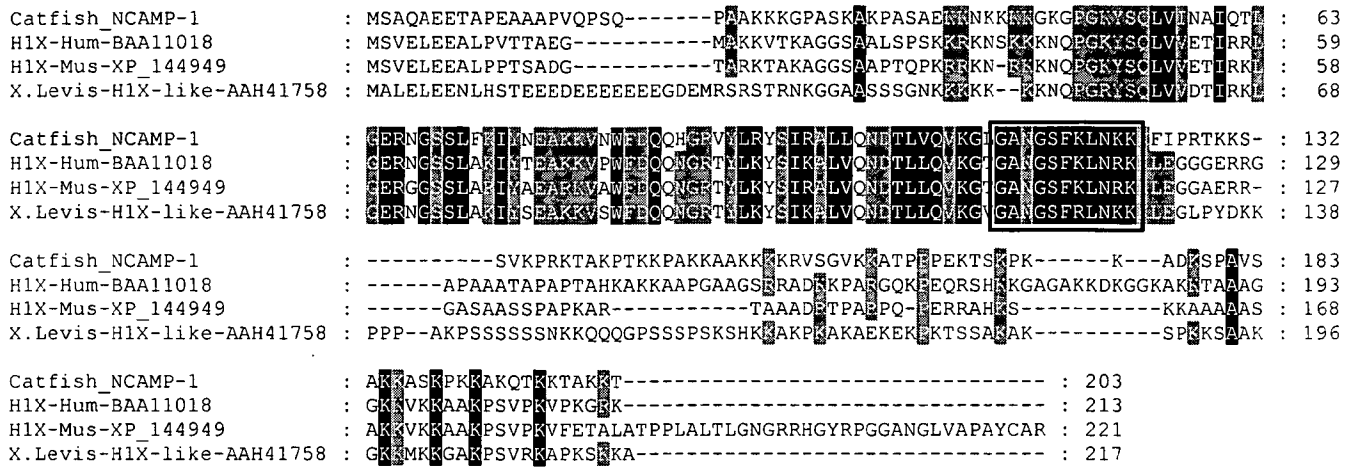

Catfish_NCAMP-1 : MSAQAEETAPEAAAPVQPSQ-------PAAKKKGPASKAKPASAEVNNKKVGKGPGKYSGLVINAIQTE : 63
H1X-Hum-BAA11018 : MSVELEEALPVTTAEG----------MAKKVTKAGGSAALSPSKKRKNSKKKNQPGKYSQLVVETIRRL : 59
H1X-Mus-XP_144949 : MSVELEEALPPTSADG----------TARKTAKAGGSAAPTQPKRRKN-KKKNQPGKYSQLVVETIRKL : 58
X.Levis-H1X-like-AAH41758 : MALELEENLHSTEEEDEEEEEEEGDEMRSRSTRNKGGAASSSGNKKKKK--KKNQPGRYSQLVVDTIRKL : 68
Catfish_NCAMP-1 : GERNGSSLFKIVNEAKKVNWFFQQHGPVYLRYSIRALLQNDTLVQVKGLGANGSFKLNKKLFIPRTKKS- : 132
H1X-Hum-BAA11018 : GERNGSSLAKIVTEAKKVPWFFQQNGRTYLKYSIKALVQNDTLLQVKGTGANGSFKLNRKLLEGGGERRG : 129
H1X-Mus-XP_144949 : GERGGSSLAKIVAEARKVAWFLQQNGFTYLKYSIRALVQNDTLLQVKGTGANGSFKLNRKLLEGGAERR- : 127
X.Levis-H1X-like-AAH41758 : GERNGSSLAKIVSEAKKVSWFFQQNGRTYLKYSIKALVQNDTLLQVKGVGANGSFRLNKKLLEGLPYDKK : 138
Catfish_NCAMP-1 : ----------SVKPRKTAKPTKKPAKKAAKKKKRVSGVKKATPLPEKTSKPK------K---ADKSPAVS : 183
H1X-Hum-BAA11018 : ------APAAATAPAPTAHKAKKAAPGAAGSKRADKKPARGQKREQRSHKKGAGAKKDKGGKAKKTAAAG : 193
H1X-Mus-XP_144949 : ------GASAASSPAPKAR----------TAAADETPAPPQ-KERRAHKS----------KKAAAAAS : 168
X.Levis-H1X-like-AAH41758 : PPP--AKPSSSSSSNKKQQQGPSSSPSKSHKKAKPKAKAEKEKKKTSSAKAK----------SPKKSAAK : 196
Catfish_NCAMP-1 : AKKASKPKKAKQTKKTAKKT---------- : 203
H1X-Hum-BAA11018 : GKKVKKAAKPSVPKVPKGKK---------- : 213
H1X-Mus-XP_144949 : AKKVKKAAKPSVPKVFETALATPPLALTLGNGRRHGYRPGGANGLVAPAYCAR : 221
X.Levis-H1X-like-AAH41758 : GKKMKKGAKPSVRKAPKSKKA---------- : 217

Fig. 3. Comparisons of the deduced amino acid sequence of catfish ncamp-1 with histone H1 proteins from different species (human, mouse, and Xenopus). Boxed area was also identified from primary sequencing and was the source for design of original degenerate primers. Multiple sequence alignment comparisons were made using CLUSTAL W.

	H1XHUM	H1XMUS	H1X-Xen	H1TRT	H1HUM
NCAMP-1	42.4	43.9	42.9	30.3	33.1
H1XHUM	--	68.5	53.6	28.6	26.7
H1XMUS	--	--	50	26.9	25.1
H1X-Xen	--	--	--	26.7	26.6
H1TRT	--	--	--	--	61.9

Table 1. Amino acid identity of catfish ncamp-1 to other histone-like proteins. HIXHUM: H1 histone family member X from human (Accession # BAA11018), H1XMUS: H1 histone family member X from mouse (Accession # XP_144949), HIX-Xen: H1 histone family member X from *Xenopus levis* (Accession # AAH41758), H1TRT: Histone H1 from trout (Accession # CAB37646), H1HUM: Histone H1 from human (Accession # P10412).

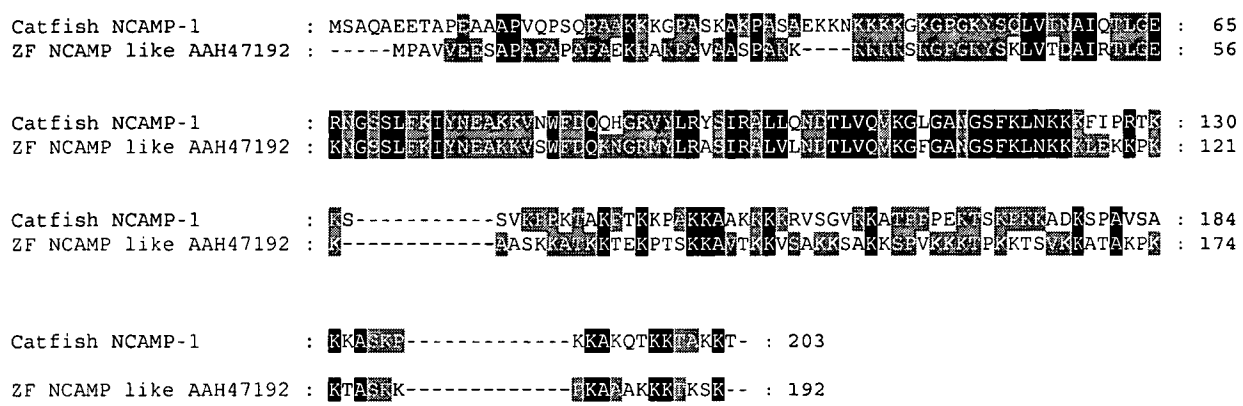


Fig 4. Comparisons of the deduced amino acid sequence of catfish ncamp-1 with a similar protein with unknown functions reported from zebrafish (*Danio rerio*). Sequence alignment comparisons were made using CLUSTAL W.

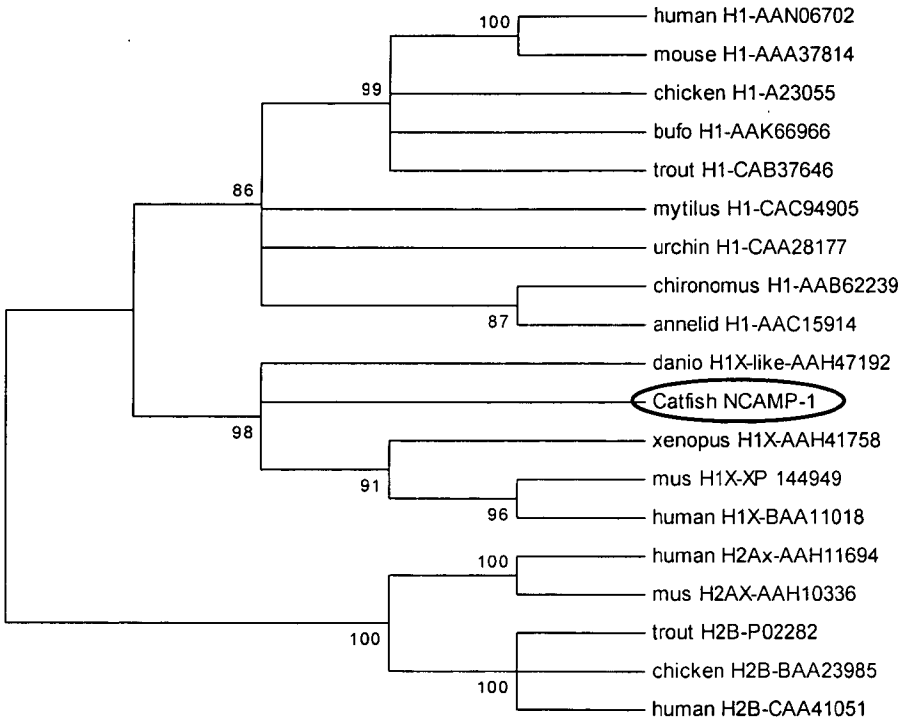
	NCAMP-1	H1XHUM	H1XMUS	H1X-Xen	H1TRT	H1HUM
Danio NCAMP- like (AAH47192)	51.2	44.1	41.3	42.9	34.7	33.9

Table 2. Amino acid identity of ncamp-1-like protein in zebrafish to other histone-like proteins. HIXHUM: H1 histone family member X from human (Accession # BAA11018), H1XMUS: H1 histone family member X from mouse (Accession # XP_144949), HIX-Xen: H1 histone family member X from *Xenopus levis* (Accession # AAH41758), H1TRT: Histone H1 from trout (Accession # CAB37646), H1HUM: Histone H1 from human (Accession # P10412).

Other data from our laboratory suggested that like histones, the ncamp-1 gene does not have introns (data not shown). The phylogenetic analysis of ncamp-1 with other histone-like proteins indicated that this protein could be a separate evolutionary branch from the histone-like protein family (Figure 5). Although ncamp-1 appears related to the histone family, more closely to H1 histone family X members, this relationship is based on the conserved central domains in these proteins. The exact physiological functions of histone H1 family X members are still not understood. Present observations will contribute towards various predicted functions for histone family members other than nuclear assembly.

Ncamp-1 was next examined for amino acid repeats or presence of conserved motifs. The periodic expression of lysine residues with boxed nonlysine spacer amino acids indicated the presence of a novel motif. The multiple lysines are repetitively arranged in boxes characterized by: KxxxK, KKxxK and KxxKK) with an apparent "preference" for alanine and proline for spacer amino acids. We refer to these repeats as lysine box motifs (LBMs). Because of the similar relationship of the new protein with histone 1X proteins from other vertebrates, we next determined whether the LBMs were conserved in any phylogenetic relationship with other proteins/peptides. In Table 3 LBMs were identified in several low mw peptides that have been previously shown to have antimicrobial activity. For comparative purposes, ncamp-1 was divided into three portions/peptides each containing regions of increased expression of LBMs (i.e. ncamp-1.1, -1.2 and -1.3). In Table 3, five non-histone antimicrobial peptides (AMP) and nine histone-like peptides from phylogenetically diverse species (e.g. bacteria to human) are compared with ncamp-1 peptides for expression and frequency of LBM repeats. Comparisons demonstrated that there were essentially no sequence identities between peptides, however there were striking similarities in expression of LBMs. From 1-5 LBM repeats were found in these

Fig.5. Phylogenetic analysis of catfish ncamp-1: Phylogram showing relationships of catfish ncamp-1 to other histone-like proteins. The tree was derived by parsimony analysis, with Mega version 2. Numbers shown above the branches are bootstrap values based upon 1000 replicates for parsimony. A separate analysis using maximum likelihood and neighbor joining methods produced a tree with similar topology. The tree was rooted on a sub-tree containing histone H2 and similar proteins.



Antimicrobial Peptides	# of LBMs	Species	Accession #
Bacteriocin : AYSLQMGATAIKQVKKLFKKW	2	Bacteria	P80214
Cecropin A : PKWKLFKKIEKVGQNIRDGIIKAGPAVA	2	Moth	M63845
Cupiennin : FKFLAKKVAKTVAKQAAKQGAK	5	Spider	P82358
ncamp-1.1 : GPASKAKPASAEKKNKKKKGKGPGKY	4	Catfish	AY324398
ncamp-1.2 : PRKTAKPTKKPAKKAAKKKKRVSG	4	Catfish	AY324398
ncamp-1.3: PKKADKSPAVSAKKASKPKKAKQTKKTAKKT	3	Catfish	AY324398
H1-Trt : AEVAPAPAAAAPAKAPKKKAAAKPKK	2	Trout	
H1-Trt : KAVAAKKSPKKAKKPAT	2	Trout	
H2B-Trt : PDPAKTAPKKGSKKVTKXA	3	Trout	
H2A-CF : KGRGKQGGKVRAKAKTRSS	3	Catfish	
H1-Trt : AEVAPAPAAAAPKAPKKA	1	Trout	
H2B-Bass1 : PEPAKSAPKKGSKKAVT	3	Sea Bass	
H2B-Bass2 : PDPAPKTAPKKGSKKAVTKTAG	4	Sea Bass	
Buforin I : AGRGKQGGKVRAKAKTRSSRAG	2	Toad	X011064
Magainin II: GIGKFLHSAKKFGKAFVGEIMNS	1	Frog	A29771
H2B/H3-Hum : KAPRKQLATPEPAKSAPAPKKGXKKXVTKA	4	Human	
H1-Human : KLNKKAASGEAKPKAKAKSPKKAKA	4	Human	

Table 3. Lysine box motifs, anti-microbial peptides and phylogeny. The expression and frequency of LBMs by AMP from diverse species is compared with three peptides from ncamp-1.

antimicrobial peptides. Examples of other AMP not shown but that also expressed multiple LBMs are adenoregulin from the leaf frog (*Phyllomedusa bicolor*: containin 3 LBMs; accession #P31107) and brevinin-2E (containing 2 LBMs; accession # S33730) from the European frog (*Rana esculenta*).

Because of the high frequency of LBM expression in AMP from diverse species from bacteria to mammals, experiments were next conducted to determine if ncamp-1 had antibacterial activity. In order to accomplish this, a recombinant form of ncamp-1 was expressed in *E. coli* and tested for antimicrobial activity in an *in vitro* assay. Recombinant ncamp-1 was expressed as described in the Materials and Methods section. The purified histidine-tagged recombinant protein had an apparent mw of 29-30 kDa identified by Western blot examination using His-Probe HRP (data not shown; Pierce, Oakbrook, IL). The apparent mw discrepancy is produced by the abundant lysine residue content of this protein. This phenomenon has been previously reported for other histone-like AMP (Hiemstra et al. 1993) that have a lower computed molecular weight compared to their (experimental) electrophoretic mobilities.

The *in vitro* bacteriocidal assay was developed using the Gram positive organism *Micrococcus luteus* as described in the Materials and Methods. Bacteria was grown overnight at 37° C, enumerated and the indicated proteins or assay buffer (media control/BSA) were mixed with 5000 CFUs in a final volume of 100 ul and incubated at 37C for 2h with shaking. Serial dilutions of each sample were plated onto MH agar plates and colony counts were determined. Figure 6 demonstrates that less than 1 ug of ncamp-1 produced approximately 50% reduction in viability 5000 CFUs of *M. leuteus*. As an additional control, recombinant ncamp-1 was also

tested at equivalent concentrations for lytic activity against catfish rbcs. Lysis was not observed (data not shown).

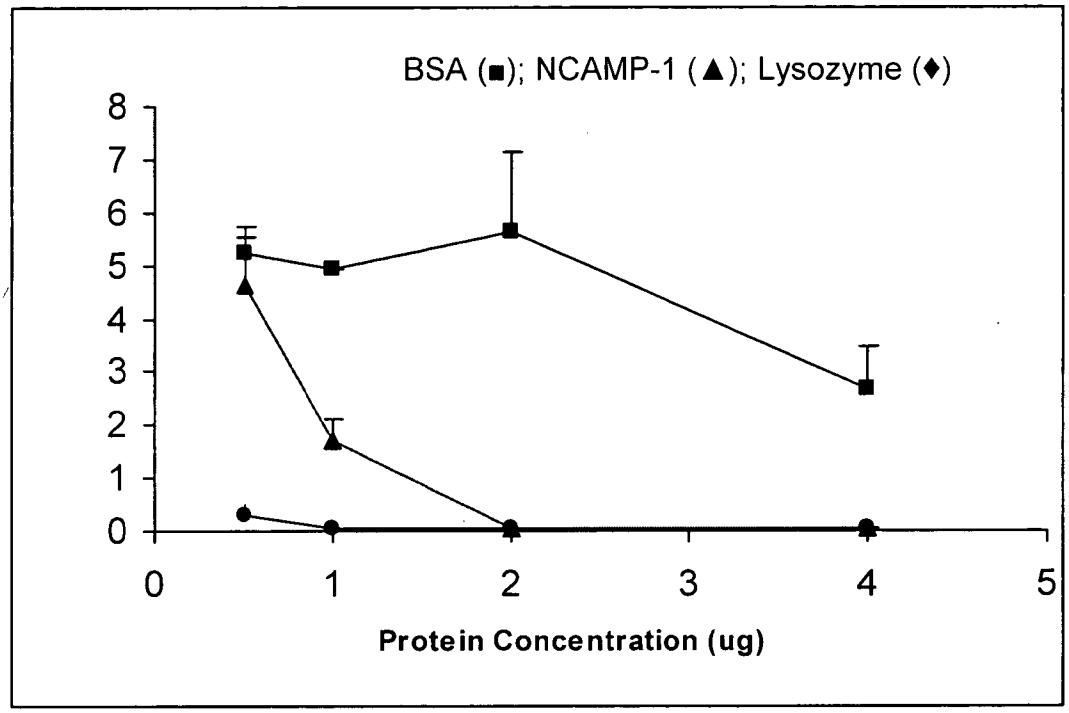


Fig 6. Recombinant ncamp-1 has anti-bacterial activity *in vitro*. Cells (*M. luteus*) were grown overnight at 37° C in Mueller-Hinton (MH) broth. Overnight cultures (500 ul) were diluted 1:100 in fresh broth and incubated for 2 hours at 37° C. After 2 hours, the OD_{620} was measured. Using a previously determined relationship of OD_{620} 0.1= 1.2 x 10^7 CFUs/ml, bacteria was diluted to 10^5 CFUs/ml in sodium phosphate buffer (pH 8) supplemented with 10% with MH broth (assay buffer). The indicated proteins or assay buffer (media control) were mixed with 5000 CFUs (in triplicate) in a final volume of 100ul and incubated at 37C for 2 h with shaking. Serial dilutions of each sample were plated onto MH agar plates and residual colony counts were determined. Colony counts were determined after overnight incubations at 37° C and were expressed as a percent of control (media) growth. Data shown are representative of at least 3 different experiments.

Thus, we now have expressed a recombinant protein that has antimicrobial activity, and determined that the LBMs contained within this protein were identical to those motifs found in other AMPs derived from evolutionarily distant species. An important question regarding these findings was: can an artificial peptide be constructed that is composed of LBMs and that has antimicrobial activity? Such a peptide has been synthesized. An artificial LBM-like containing peptide has been designed and expressed by others. It is a leucine and lysine (LK) copolymer (Beven et al. 2003). The copolymer with the greatest anti-bacterial activity against cell wall-less *Mollicutes* (acholephasmas, mycoplasmas, etc.) was the 15-mer peptide KLL**K**LLL**K**LLL**K**LLK. This LBM containing peptide was predicted to assume an alpha-helical comformation in aqueous solution; it was amphipathic; and the predicted mechanism of bactericidal action was based on the interfacial model of "carpet" attachment followed by induced structural changes in the bacterial cell lipid bilayer. This was followed by cell death caused by depolarization of the bacterial cell membrane (Beven et al. 2003). These data clearly supported the hypothesis that the required structure in an AMP to elicit bacteriocidal activity is an LBM and that this repeat is phylogenetically conserved.

The analysis of ncamp-1 structure and function demonstrated its phylogenetic relationship to AMPs from both prokaryotes and eukaryotes. The genetic relatedness of ncamp-1 to histones goes beyond the abundant presence of lysines and its ability to bind DNA.

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Anti-bacterial Activity of Soluble Recombinant NCAMP-1

Soluble recombinant NCAMP-1 has been shown in an anti-bacterial assay to kill both a gram negative (*E. coli*) and gram positive (*Micrococcus luteus*) bacteria. The killing capacity of NCAMP-1 against clinical isolates of a fish pathogen, *Streptococcus iniae* has been studied. Growth and anti-bacterial assay conditions have been established for 2 of these isolates. Results indicate strain dependent sensitivity to the antibacterial activity of NCAMP-1. (ie. Isolate KFP164 is susceptible while isolate DAN 14 is completely resistant). Preliminary characterization of 10 additional clinical isolates and a reference strain (ATCC) similarly reflect strain dependent sensitivity to NCAMP-1 activity.

NCAMP-1 Truncation Constructs.

Rationale . Analysis of hydrophobicity plots of NCAMP-1 together with known H1 domain structures indicate a tripartite molecule with a central hydrophilic helical domain flanked by charged hydrophobic domains. We determined which of these domains are necessary (sufficient) for antimicrobial activity and DNA binding activities.

Inspection of NCAMP-1 sequence indicated the following constructions for testing:

- NCAMP-1 – Full length, amino acids 1-203 (shown to have anti-microbial activity as indicated above)
- NCAMP-d(deletion)1 –Amino acids 1-118; (deletion of the C-terminal charged, hydrophobic domain leaving the N-terminal charged hydrophobic and central hydrophilic domains).
- NCAMP-d(deletion)2 –Amino acids 1-60 (deletion of both the central and C-terminal domains leaving only the N-terminal charged hydrophobic domain).

PCR was used to generate restriction sites for insertion of constructs into the BamHI-XhoI site of pET 21b (Novagen). Ligation into this site results in expression plasmids with a C-terminal 6xhis tag. Plasmids were initially transformed into *E. coli* strain DH5a for stock plasmid production before transforming into the IPTG-inducible E. coli expression strain BL21(DE3) pLysS (Novagen). Cultures were induced after growth to OD_{600} 0.6-1 and cells were harvested 3 hours later. Cell lysates were prepared by treatment with lysozyme, Triton X-100, DNaseI and RNaseA. Recombinant protein was recovered with nickel chelating resin (NINTA agarose, Qiagen) according to the manufacturers protocol.

Testing of these constructs indicates that the d2 constuct, but not the d1 is capable of anti-bacterial activity against *E. coli*. Further, the dose response curve of d2 activity appears similar to that of full length NCAMP-1. In contrast, DNA binding activity (as determined by ODN blotting experiments) is completely abolished in both d1 and d2 constructs indicating the C-terminal domain is necessary for DNA binding activity.

Abstract

Nonspecific cytotoxic cells (NCC) are the first identified and most extensively studied killer cell population in teleosts. NCC kill a wide variety of target cells including tumor cells, virally transformed cells and protozoan parasites. NCC may participate in innate immune responses by the indirect killing of bacteria. In the present study a novel evolutionarily conserved NCC membrane protein was sequenced from a channel catfish (*Ictalurus punctatus)* NCC cDNA library using primers designed based on the MS analysis of tryptic digests from a DNA binding surface protein from NCC. The protein has a computed molecular weight of 22,064.63 Daltons and a basic pI (10.75); it is lysine rich; and amphipathic. Sequence comparisons of this protein by various methods indicated a close similarity to zebrafish, trout, mouse and human H1 histone family member X. This was confirmed by phylogenetic analysis. Physiological functions of this protein were evaluated by expressing it in a recombinant form. This novel protein seems to bind to DNA and possess antibacterial activity, which was confirmed by *in vitro* killing of *Micrococcus luteus*. The novel protein is referred to as NCC antimicrobial protein-1 (ncamp-1). Expression of ncamp-1-like proteins in various tissues of channel catfish as well as zebrafish was verified by searching EST databases. Inspection for signature repeats in ncamp-1 indicated the presence of multiple lysine box motifs (LBMs) composed of KxxxK, KKKK, KKxxK or KxxKK. Naturally occurring low mw (17-30 aa) antimicrobial proteins from phylogenetically diverse organisms (bacteriocin from *Lactobacillus plantarum*; cecropin A from moth; cupeinin from spider; buforin I from toad; histone 2B from human) also contained LBMs at a much greater frequency than those occurring in ncamp-1. The present study indicates that ncamp-1 of channel catfish NCC is composed of phylogenetically conserved motifs that are also found in other anti-microbial proteins/peptides produced in

evolutionary distant species. The presence of these motifs in a peptide may be predictive of antibacterial activity.

Key Words. Nonspecific cytotoxic cells (NCC); antimicrobial proteins, oligodeoxynucleotides, lysine box motif, *Micrococcus luteus*.